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Age-related changes in surface antigens on peripheral lymphocytes of healthy children

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SUMMARY

The age-related changes in proportion of various subsets within lymphocytes were investigated in cord blood and peripheral blood from healthy children and adults. The percentages of T and B cells did not show age-related changes, whereas natural killer (NK) cells increased significantly with age. Within lymphocytes or the $CD3^+$ T cell population the proportion of $CD45RA^{bright+}$ lymphocytes decreased and that of $CD45RO^+$ cells increased, while that of $CD45RA^{dim+}$ cells showed no age-related change. Within lymphocytes, the percentage of $CD45RA^{bright+}$ $CD4^+$ cells decreased, together with a decline of that of $CD4^+$ cells. The proportions of $CD45RA^{bright+}$ $CD8^+$ cells and $S6F1^{bright+}$ $CD8^+$ cells increased with age, and the age-dependent increase of the proportion of $CD8^+$ cells seems to be mainly attributable to the increases in these subsets. The $CD45RA^{dim+}$ $CD4^+$ and $CD45RA^{dim+}$ $CD8^+$ cells co-expressing CD45RO at a low level nevertheless showed no age-related changes. In $\gamma\delta$ T cells, both $\delta TCS1^+$ and $\delta TCS1^-$ T cells increased with age, but the $\delta TCS1^-\gamma\delta$ T cells increased more than the $\delta TCS1^+$ subset. Among lymphocytes, the percentages of $CD20^+$, $CD21^+$ and $CD22^+$ cells remained similar, with no age-related changes, but the proportion of $CD5^+$ cells within lymphocytes or B cells decreased. The proportions of $CD16^+$ NK cells among lymphocytes increased with age, and this change was attributable to the increase of $CD56^+$ cells.

Keywords CD45RA CD45RO lymphocyte subset children

INTRODUCTION

Recently multi-colour flow cytometric analysis has been introduced to analyse precisely the surface markers of peripheral lymphocytes, and to identify the numerical deficiency of various subpopulations [1,2]. A good understanding of the process of immune development is necessary to evaluate immune status, and these subpopulations change with age in normal infants and adults. However, only some of these age-related changes of lymphocyte subsets, including $CD45RO^+$ or $CD45RA^+$ $CD4^+$ cells, $CD5^+$ B cells and natural killer (NK) cells have so far been well established [1–7]. In this study we evaluated the age-related changes in the all T, B and NK cell populations by identifying a total of 15 subsets of lymphoid cells in healthy children. Our data show significant age-related changes in the proportions of $CD45RA^{bright+}$ ($CD45RA^{bri+}$) and $CD45RO^+$ T cells, $S6F1^{bright+}$ ($S6F1^{bri+}$) $CD8^+$ cells, $\gamma\delta$ T cells, $CD5^+$ B cells and NK cells. These changes may relate to the development of immune competence, perhaps under the influence of exposure to extrinsic antigens.

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MATERIALS AND METHODS

Samples

We analysed the surface markers of cord blood samples obtained from eight neonates born at 37–42 weeks of gestation and peripheral blood samples from 11 infants, 16 healthy children aged 1–18 years, and six healthy adults aged 20–25 years. Cord blood was collected immediately after uneventful delivery by healthy mothers. Cord and venous blood from the infants and children were collected after obtaining informed parental consent. Adult venous blood was obtained from volunteers, i.e. the staff of the university hospital.

Lymphocyte preparation and flow cytometric analysis

Mononuclear cells were isolated from heparinized whole blood by Ficoll–Hypaque gradient centrifugation. Residual erythrocytes were lysed with ammonium chloride. In order to prevent non-specific binding of MoAbs to Fc receptors, cells were blocked with human gamma globulin for 10 min. For two-colour analysis, cells were suspended at a concentration of 5×10^5 cells/50 μl in buffer (PBS/0·2% bovine serum albumin (BSA)/0·1% NaN_3) and incubated with FITC- or

Table 1. Combinations of MoAbs used in the two- and three-colour immunofluorescence assays

	FITC	PE	perCP
T cell subsets	CD4	CD45RA	
	CD8	CD45RA	
	CD3	CD45RA	
	CD45RO	CD3	
	CD8	S6F1	
	TCRδ1	CD3	
	δTCS1	CD3	
	CD45RO	CD45RA	CD3, CD4, or CD8
B cell subsets	CD20	CD21	
	CD20	CD5	
	CD23	CD20	
NK cell subset	CD16	CD56	

NK, Natural killer.

PE-conjugated mouse anti-human MoAbs in a dilution of 1:500 for 15 min at 4°C. For three-colour analysis, perCP-conjugated MoAbs were added. For single-colour analysis, cells were incubated with unconjugated MoAbs for 15 min at 4°C. After two washes with buffer, the cells were incubated with FITC-labelled rabbit anti-mouse immunoglobulin $F(ab')_2$ fragments (Dako, Kyoto, Japan) [8]. Stained cells were washed twice and analysed using FACScan flow cytometry equipped with Consort 30 and Paint-a-gate research softwares. For each sample, only lymphocytes were analysed by electrical gating, and 7000 cells were analysed on a logarithmic scale for fluorescence.

The MoAbs used were: FITC- or PE-labelled T3 (CD3), B1 (CD20), T4 (CD4), T8 (CD8), 2H4 (CD45RA), S6F1 (CD11a), B2 (CD21) and NKH1 (CD56) (Coulter Immunology, Hialeah, FL); TCRδ1, δTCS1 (T Cell Sciences, Cambridge, MA); Leu-11 (CD16), Leu-4 (CD3), Leu-3 (CD4), Leu-2a (CD8) and Leu-1 (CD5) (Becton Dickinson, Mountain View, CA); and UCHL1 (CD45RO) and H107 (CD23) (Nichirei Co., Tokyo, Japan). The combinations of MoAbs for two-colour analysis used in this study are shown in Table 1. For three-colour analysis, perCP-conjugated Leu-4, Leu-3, and Leu-2 were used. For single-fluorescence assay, B3(CD22) (Coulter) was used.

Table 2. Coefficient of correlation between age and the proportions of given subsets

	r	*P*
Within lymphocytes		
$CD4^+$	−0·63	<0·01
$CD8^+$	0·49	<0·01
CD4/CD8 (ratio)	−0·53	<0·01
$CD45RA^{bri+}CD3^+$	−0·65	<0·01
$CD45RA^{dim+}CD3^+$	−0·06	NS
$CD45RO^+CD3^+$	0·75	<0·01
$CD45RA^{bri+}CD4^+$	−0·77	<0·01
$CD45RA^{bri+}CD8^+$	0·41	<0·05
$CD45RA^{dim+}CD45RO^{dim+}CD4^+$	−0·19	NS
$CD45RA^{dim+}CD45RO^{dim+}CD8^+$	0·26	NS
$S6F1^{bri+}CD8^+$	0·59	<0·01
δTCS1	0·40	<0·05
TCRδ1	0·59	<0·01
δTCS1/TCRδ1 (ratio)	−0·31	NS
Within $CD3^+$ lymphocytes		
$CD45RA^{bri+}$	−0·75	<0·01
$CD45RA^{dim+}$	−0·13	NS
$CD45RO^+$	0·70	<0·01
Within $CD4^+$ lymphocytes		
$CD45RA^{bri+}$	−0·71	<0·01
Within $CD8^+$ lymphocytes		
$CD45RA^{bri+}$	0·12	NS
$S6F1^{bri+}$	0·28	NS

r, Coefficient of correlation; NS, Not significant.

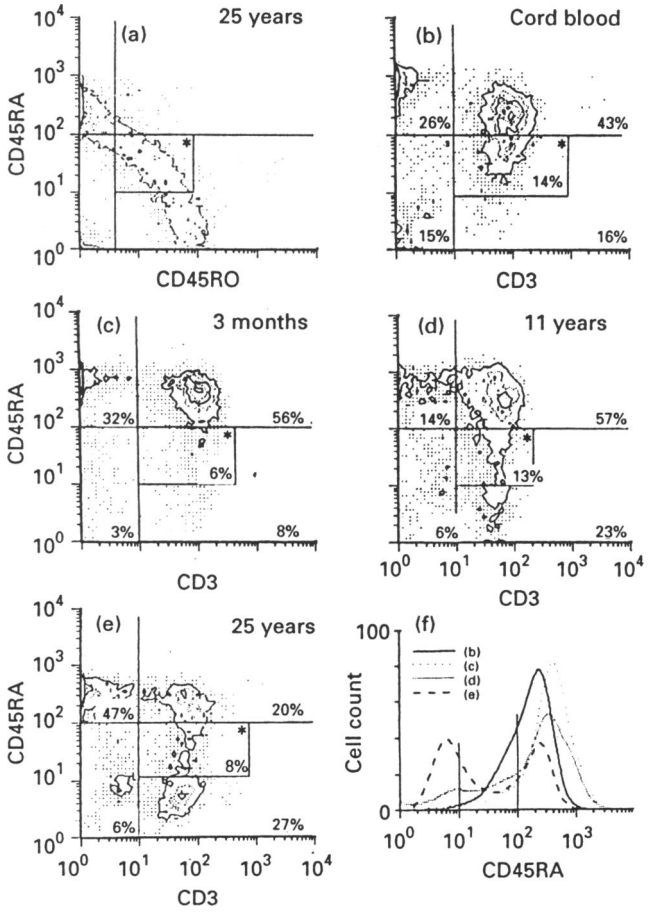

Fig. 1. Representative expression patterns of CD45RA and CD45RO on blood lymphocytes using two-colour analysis: CD45RO with CD45RA MoAbs on cells from 25-year-old person (a), and CD3 (abscissa) with CD45RA (ordinate) on cells from cord blood (b), and peripheral blood of 3-month-old (c), 11-year-old (d), or the same person as (a) (e). An overlay histogram of $CD3^+$ cells in b–e. (f) The gated cells shown were small lymphocytes including approximately 10% $CD3^-CD45RA^+$ B lymphocytes and some natural killer (NK) cells. * $CD45RA^{dim+}$ cells.

RESULTS

T, B and NK cells

The percentages of T (CD3), B (CD20) and NK cells (CD56) among lymphocytes were 66·1 ± 7·0%, 18·3 ± 5·2% and 10·3 ± 6·1% (mean ± s.d.), respectively. The percentage of NK cells (CD56) increased with age ($r = 0·34$, $P < 0·05$), whereas no significant changes were observed in the proportions of T and B cells.

T cell subsets

The correlations between age and the proportion of T cell subsets within lymphocytes are summarized in Table 2. The percentage of $CD4^+$ cells decreased, while that of $CD8^+$ cells increased with age. The CD4/CD8 ratio was high in infancy (2·4 ± 0·6 (mean ± s.d.)) and decreased with age.

When co-labelled with an anti-CD45RA MoAb, $CD3^+$ lymphocytes were classified into three subsets: highly positive ($CD45RA^{bri+}$), dimly positive ($CD45RA^{dim+}$) and negative ($CD45RA^-$). Three-colour analysis showed that CD45RO was expressed in inverse proportion to CD45RA expression. The $CD45RA^{bri+}$ cells tended to be $CD45RO^-$ and *vice versa* (Fig. 1a). A $CD45RA^{dim+}$ subset was also identified: these cells were $CD45RO^{dim+}$. The proportion of $CD45RA^{bri+}CD3^+$ cells within lymphocytes decreased significantly ($r = -0·65$, $P < 0·01$), while that of $CD45RO^+CD3^+$ cells increased with age ($r = 0·75$, $P < 0·01$) (Fig. 2). Among $CD3^+$ cells, the proportion of $CD45RA^{bri+}$ cells was higher, and that of $CD45RO^+$ cells was lower in cord blood and infancy compared with greater ages. The percentage of $CD45RA^{dim+}CD45RO^{dim+}CD3^+$ cells within lymphocytes showed no change with age (10·6 ± 4·4% (mean ± s.d.)) (Fig. 2). The percentage of $CD45RA^{dim+}CD45RO^{dim+}CD4^+$ or $CD8^+$ lymphocytes showed no change with age ($CD45RA^{dim+}CD45RO^{dim+}CD4^+$, $r = -0·19$; $CD45RA^{dim+}$ $CD45RO^{dim+}$ $CD8^+$, $r = 0·26$). The proportion of

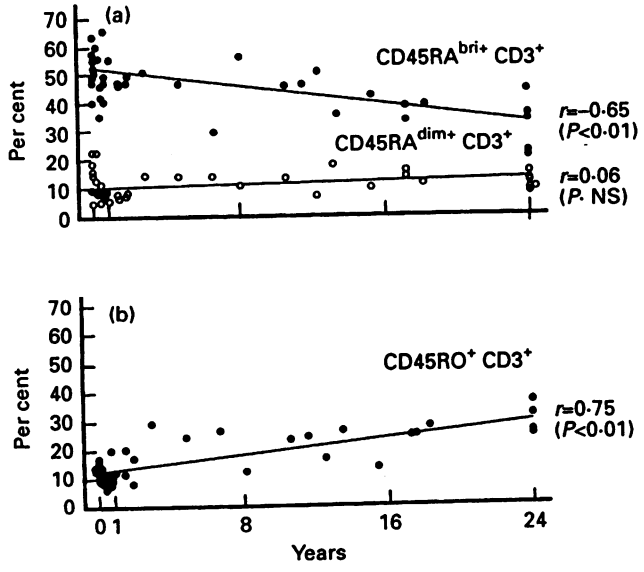

Fig. 2. The relationship between age and the percentage of $CD45RA^{bri+}$ cells (●), $CD45RA^{dim+}$ cells (○) (a), or $CD45RO^+$ cells (b), within lymphocytes.

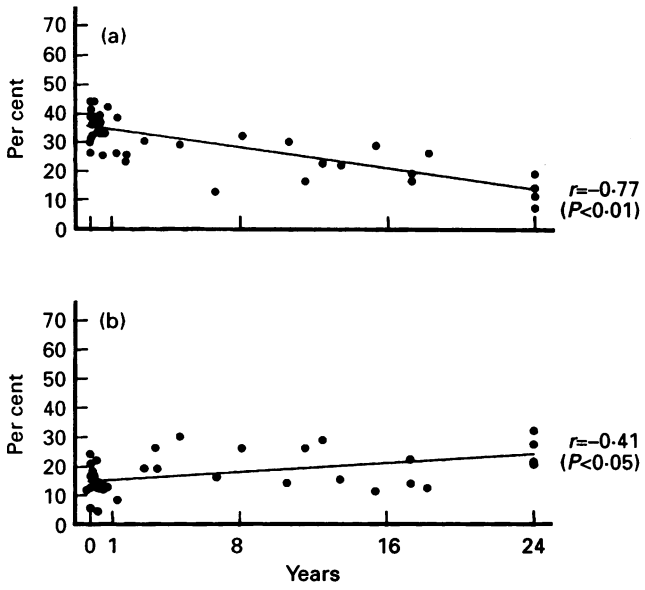

Fig. 3. The relationship between age and percentage of $CD45RA^{bri+}CD4^+$ cells (a), or $CD45RA^{bri+}CD8^+$ cells (b) within lymphocytes.

$CD45RA^{dim+}CD45RO^{dim+}CD3^+$ was high in cord blood. Figure 1 shows a temporary dip in $CD45RA^{dim+}CD45RO^{dim+}$ cells at 3 months old and stability afterwards. We also investigated the $CD45RA^{bri+}CD4^+$ and $CD45RA^{bri+}CD8^+$ subsets. The percentage of $CD45RA^{bri+}CD4^+$ lymphocytes decreased in parallel with the age-related decrease in $CD45RA^{bri+}CD3^+$ lymphocytes ($r = -0·77$, $P < 0·01$), and that of the $CD45RA^{bri+}$ cells among $CD4^+$ cells also decreased. Although $CD45RA^{bri+}CD8^+$ cells among lymphocytes increased with age ($r = 0·41$, $P < 0·05$), $CD45RA^{bri+}$ cells among $CD8^+$ cells showed no age-related change, indicating that the increase in $CD8^+$ cells was partly due to the increase in the $CD45RA^{bri+}CD8^+$ subset (Fig. 3).

The S6F1 MoAb reacts with an epitope on the LFA-1α chain and $S6F1^{bri+}CD8^+$ lymphocytes mediate cytotoxic activity [9]. This subset increased with age ($r = 0·59$, $P < 0·01$) (Fig. 4). Again, the proportion of $S6F1^{bri+}$ lymphocytes among $CD8^+$ cells did not show age-related changes.

γδ T cells were identified by their dual positivity for CD3 and TCRδ1. $TCR\delta1^+CD3^+$ cells showed a significant increase with age ($r = 0·59$, $P < 0·01$). Although a subset of γδ T cells,

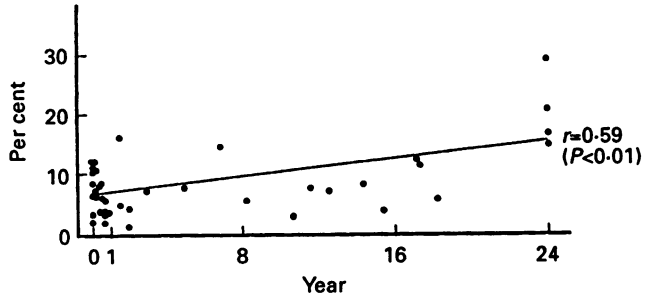

Fig. 4. The relationship between age and percentage of $S6F1^{bri+}CD8^+$ cells within lymphocytes.

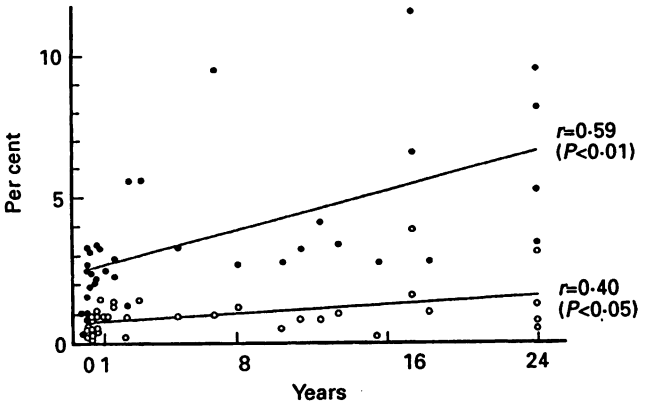

Fig. 5 The relationship between age and percentage of TCRδ1$^+$CD3$^+$ cells (●) and δTCS1$^+$CD3$^+$ cells (○) within lymphocytes.

δTCS1$^+$CD3$^+$ cells expressing Vδ1, also increased with age ($r = 0{\cdot}40$, $P < 0{\cdot}05$), δTCS1$^-$ cells increased more than the δTCS1$^+$ T cells, indicating a predominant increase of $\gamma\delta$ T cells expressing Vδ2 (Fig. 5).

B cell subsets

The percentages of CD20$^+$, CD21$^+$ and CD22$^+$ cells among the lymphocyte populations were similar, indicating that these reagents identified similar B lymphocyte populations (data not shown).

A significant inverse correlation with age was observed for the proportion of CD5$^+$CD20$^+$ cells among lymphocytes ($r = -0{\cdot}42$, $P < 0{\cdot}05$), as well as the CD5$^+$ cells within the CD20$^+$ B lymphocyte count ($r = -0{\cdot}59$, $P < 0{\cdot}01$) (Table 3a). The proportion of CD5$^+$ cells was the highest among the B cells seen in the cord blood ($0{\cdot}78 \pm 0{\cdot}13$ (mean ± s.d.)), and then gradually decreased with age to a $0{\cdot}37 \pm 0{\cdot}13$ value in adults (> 20 years old). CD23$^+$CD20$^+$ cells showed no age-related changes (Table 3a); among the CD20$^+$ subset, the proportion of CD23$^+$ lymphocytes remained about $0{\cdot}51 \pm 0{\cdot}15$.

Table 3. Coefficient of correlation between age and the proportions of given subsets

a. B cell subsets

	r	*P*
Within lymphocytes		
CD5$^+$CD20$^+$	−0·42	< 0·05
CD23$^+$CD20$^+$	−0·06	NS
Within CD20$^+$ lymphocytes		
CD5$^+$	−0·59	< 0·01
CD23$^+$	0·25	NS

b. NK cell subsets

	r	*P*
Within lymphocytes		
CD16$^+$CD56$^+$	0.49	< 0·01
Within CD56$^+$ lymphocytes		
CD16$^+$	0·15	NS

r, Coefficient of correlation; NS, not significant; NK, natural killer.

NK cell subsets

CD16$^+$CD56$^+$ cells, the dominant NK cell subset, increased with age ($r = 0{\cdot}49$, $P < 0{\cdot}01$) in parallel with the increase in NK cells. CD16$^+$ lymphocytes within the CD56$^+$ cells showed no change with age, and mean value was $0{\cdot}70 \pm 0{\cdot}19$ (Table 3b).

DISCUSSION

The CD45RA$^+$ and CD45RO$^+$ T cell subsets are believed to be naive and memory T cells, respectively, since CD45RA$^+$ T cells are converted into CD45RO$^+$ T cells after antigenic stimulation, and the latter subset provides a recall response to antigens [10–19]. In earlier reports, the expression patterns of CD45RA and CD45RO were analysed for the CD4$^+$ subset, and the CD45RA$^+$ subset, including both CD45RA^{bri+} and CD45RA^{dim+} cells, was studied [6,7,16]. We analysed the age-related changes in CD45RA^{bri+} and CD45RA^{dim+} T cells separately, since the function of CD45RA^{dim+} T cells was suggested to be distinct from that of CD45RA^{bri+} T cells [20–23], and this subset co-expresses CD45RO at a low level. In addition, the expression patterns of CD45RA and CD45RO were analysed not only for the CD4$^+$ subset but also for the CD8$^+$ subset. CD45RA^{bri+}CD3$^+$ lymphocytes decreased with age, as expected on the basis of previous observation [3,6,7], while no age-related changes in CD45RA^{dim+} T cells were detected. This observation supports the hypothesis that CD45RA^{bri+} and CD45RA^{dim+} T cells mediate different functions. Interestingly, the proportion of CD45RA^{dim+} CD45RO^{dim+}CD3$^+$ lymphocytes was already high in cord blood, with a termporary decrease in number around 3 months of age. This subset increased after 3 years of age (Fig. 1b–f). Bofill *et al.* recently reported that CD45RA^{dim+}CD45RO^{dim+} T cells in cord blood acquired high levels of CD45RA after coculture with fibroblasts, and suggested that these cells are antecedents of CD45RA^{bri+} cells [23]. However, it is still unclear whether CD45RA^{dim+} T cells in children and adults are precursors of CD45RA^{bri+} cells.

Although there is a report by Froebel *et al.* that the percentage of CD45RO$^+$CD8$^+$ cells is constant during the period between 1 and 6 years of age [4], previous observations about CD45RA and CD45RO expression within the CD8$^+$ subset during ontogeny have been particularly scanty. In this study, a positive correlation of CD45RA^{bri+}CD8$^+$ cells with age was observed, and this correlation was in contrast to that of CD45RA^{bri+}CD4$^+$ cells. Although it has been suggested that CD45RO$^+$CD8$^+$ lymphocytes are a memory cytotoxic population [12,24–27], our present findings show that only a few of these cells circulate in the peripheral blood.

S6F1 recognizes an epitope of the LFA1α-chain molecule, and S6F1^{bri+}CD8$^+$ lymphocytes were reported to be cytotoxic T cells [9]. This subset showed a positive correlation with age. However, the proportions of S6F1^{bri+} cells and CD45RA^{bri+} cells among CD8$^+$ lymphocytes did not change with age. Consequently, age-related increase in CD8$^+$ cells seems to be mainly attributable to the increase in S6F1^{bri+} and CD45RA^{bri+} subsets.

It was reported that $\gamma\delta$ T cells increased with age in the peripheral blood, peaking at around 10 years of age [28,29].

However, our data showed that $\gamma\delta$ T cells continued to increase with age until at least 25 years old. In $\gamma\delta$ T cells, $\gamma\delta$ T cells expressing Vδ1 (δTCS1$^+$CD3$^+$) are predominant in the thymus. δTCS1$^-$ $\gamma\delta$ T cells, including those which mainly express Vδ2, are predominant in the peripheral blood [28], and were suggested to proliferate mainly outside the thymus [30–36]. In this study, a more predominant increase with age was observed for δTCS1$^-$ $\gamma\delta$ T cells than for δTCS1$^+$ $\gamma\delta$ T cells, indicating that the increase in $\gamma\delta$ T cells is mainly due to the increase in the numbers of $\gamma\delta$ cells accumulating in the peripheral blood.

The proportions of B cell subsets showed no changes related to age. In this study, the proportion of CD20$^+$ cells was similar to that of CD21$^+$ cells as well as that of CD22$^+$ cells, indicating that most B cells in the peripheral blood express all three markers [37–40]. CD5$^+$CD20$^+$ lymphocytes are functionally different from conventional CD5$^-$ B cells, as they produce natural antibodies with low intrinsic affinity, as well as poly-reactive antibodies to a variety of self-antigens [41,42]. This subset was larger at younger ages than in adults, reflecting the immaturity of the imune system in childhood.

The percentage of NK cells (CD56$^+$ cells) was shown to increase with age as in previous studies [1,2]. Their CD16$^+$ major subset also increased with age, but the proportion of CD16$^+$ cells in CD56$^+$ cells did not show age-related changes. Therefore, the importance of NK cell mediating functions may also increase with age.

In conclusion, we have shown significant age-related changes in the proportions of CD45RA$^+$ and CD45RO$^+$ T cells, S6F1$^+$CD8$^+$ T cells, $\gamma\delta$ T cells, CD5$^+$ B cells and NK cells. Although we did not evaluate absolute numbers of these subsets, it is known that the absolute numbers of lymphocytes increase with age until 6 months old (mean: from $5{\cdot}5\times10^3$ to $7{\cdot}3\times10^3/\mu l$) and gradually decrease (mean at 21 years old: $2{\cdot}5\times10^3/\mu l$) [43]. Therefore, it seems that the absolute number of CD45RA^{bri+}CD4$^+$ cells, whose proportion declines with age, markedly decreases with age. On the other hand, absolute numbers of the subsets, whose proportion slightly increases with age, including CD45RA^{bri+}CD8$^+$ cells and δTCS1$^+$ $\gamma\delta$ T cells, appear to decrease with age, and those of CD45RO$^+$ T cells, S6F1$^+$CD8$^+$ cells, TCRδ1$^+$ $\gamma\delta$ cells and CD16$^+$ NK cells, appear to be rather constant at all ages. When evaluating the immune status of children, consideration of age-related changes is essential, and the findings of this study are important for interpreting changes in various diseases, including infections which occur in infants.

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